

Enhancement of Influenza Virus Infections by Secalonic Acid D

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Secalonic acid D (SAD), a hepatotoxic, teratogenic, and slightly mutagenic metabolite of *Penicillium oxalicum* has been identified as a natural contaminant of grain dust. Secalonic acid D was administered intraperitoneally to male ICR mice that were exposed to influenza virus aerosols 5 days earlier. The mortality rate was significantly higher ($p < 0.001$) in mice subjected to both influenza and SAD than those subjected to influenza alone. Virus titers in lung tissue samples at selected time intervals appeared similar for both influenza and influenza-SAD treated groups of mice for 9 days after exposure to the virus. After 9 days, influenza-SAD treated mice appeared to have higher virus titers. No difference in the pathological progression of pneumonia was discernible between these two groups of mice. The influenza-SAD group, in addition to pneumonia, exhibited severe hepatic necrosis characteristic of SAD administration. Mice infected with influenza virus followed by administration of SAD responded with significantly lower ($p < 0.05$) antibody titers to influenza virus than mice exposed to influenza virus alone.

Introduction

Secalonic acid D (SAD) is a metabolite of *Penicillium oxalicum* (1) and *Aspergillus aculeatus* (2). *Penicillium oxalicum* isolated from grain crops intended for consumption by domestic animals and humans has been associated with adverse effects in animals consuming contaminated grains (3-5). Detectable quantities of SAD were present in grain dust samples collected from terminal grain elevators located in the lower Mississippi River region (6). Grain dusts contaminated with SAD pose potential health hazards for workers from occupational exposure to the dust, and for animals given feed composed of pelleted grain dust.

Secalonic acid D has been reported as having toxic effects in laboratory animals. The LD₅₀ for male ICR mice administered SAD intraperitoneally (IP) was calculated to be 31 mg/kg with deaths occurring between 2 and 8 days (4,7). Following the intravenous injection of SAD, the mycotoxin was found to concentrate in the liver and kidneys (8). Pathological examinations revealed that SAD injected IP caused adhesions and peritonitis associated with the diaphragm, stomach, intestines, abdominal wall, liver, and kidneys (4,7). Hepatic portal and parenchymal necrosis was also evident. In another study,

it was reported that pulmonary atelectasis, pulmonary vascular congestion, and hemorrhaging occurred in mice following the IP injection of SAD (5,7). In addition, Sorenson et al. (9) observed lung abnormalities in rats when SAD was administered intratracheally.

This study was initiated to discern the role mycotoxin exposure might have in the progression of an infectious disease in livestock and humans. The mycotoxin SAD was chosen because it was found as a natural contaminant in grain dust and reported to be a causative agent in lung pathology. Therefore, the purpose of this study was to determine the effect of administering SAD to laboratory mice previously exposed to aerosols of influenza virus.

Materials and Methods

Experimental Animals

Animals used in this study were male ICR mice (King Laboratories, Middleton, WI) weighing between 25 and 30 g each. Mice were maintained in temperature-controlled rooms at 18°C with 12 hr light/dark cycles. Pelleted feed and fresh water were available *ad libitum*. Animals were acclimated for 1 week prior to treatment.

Influenza Virus

The Aichi (H₂N₃) strain of influenza virus was furnished by Dr. Ken Soike (Tulane University, Delta Regional Primate Center, Covington, LA). A pool of virus was prepared by one passage in embryonated chicken

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Table 1. Experimental groups.

Experimental group	Treatment	
	Day 0	Day 5
Untreated control	—	—
DMSO (vehicle only)	—	DMSO/bicarbonate*
SAD only	—	SAD ^b
Flu only	Aerosol exposure	—
Flu/DMSO	Aerosol exposure	DMSO/bicarbonate
Flu/SAD	Aerosol exposure	SAD

* 0.1 mL of 10% DMSO in 5% sodium bicarbonate.

^b 35 mg/kg body weight administered IP.

eggs followed by harvesting the allantoic fluids. The allantoic fluid (stock virus) titered $10^{-7.2}$ egg infectious doses (EID₅₀)/mL and was stored at -70°C until used.

Aerosol Exposure

A modified Henderson aerosol apparatus similar to that previously described by Akers et al. (10) was used for the aerosol exposure of mice to influenza virus. A DeVilbiss type 40 atomizer was used to aerosolize the virus suspension. The atomizer containing 5.5 mL of a 1:50 dilution of the virus suspension in Minimal Essential Medium (MEM) (Flow Laboratories, McLean, VA) was operated under 10 lb of air pressure and at a constant air flow of 12.5 L/min. Mice, in groups of 20, were exposed for 5 min receiving a dose of 366 EID₅₀ units. An AGI-30 impinger was operated continuously during the exposure periods. All aerosol runs were conducted with the aerosol apparatus operating within a Class II, Type B biological safety cabinet.

Secalonic Acid D Preparation and Administration

Flasks of yeast extract (2%)–sucrose (5%) medium were inoculated with *Penicillium oxalicum* (NRRL 5209). Secalonic acid D was extracted from these cultures according to the method of Ciegler et al. (5). Purified SAD was dissolved in DMSO and further diluted with 5% aqueous sodium bicarbonate to yield a final DMSO concentration of 10%. Throughout this study, a dose of 35 mg SAD/kg of body weight was administered IP in a volume not to exceed 0.1 mL of SAD solution/30 g of body weight. The SAD solution was injected IP 5 days after the mice were exposed to influenza virus aerosols. The DMSO control mice received 0.1 mL of DMSO diluted in 5% aqueous sodium bicarbonate to give a final concentration of 10% DMSO. The experimental regime employed is depicted in Table 1.

Mortality Studies

Mortality rate and the time of death were determined for the following groups: Flu, Flu/SAD, SAD, and Flu/DMSO. A group of untreated mice was maintained as controls. Mice were observed twice a day for up to 14 days after aerosol exposure to influenza virus. The harmonic mean time of death of each of the four groups was

determined and compared using the Kruskal-Wallis test (11). The chi-square test for two independent variables was used to determine if SAD had a significant effect on the number of deaths from influenza infection.

Viral Pathogenesis

Virus replication in the lungs of mice in the four groups as well as from DMSO and untreated control groups were determined in lungs removed aseptically on days 0, 3, 9, and 12 following aerosol exposure. Lung tissues from each group (four mice per group at each time interval), were pooled, homogenized, and suspended in MEM with 10% fetal bovine serum to give a final 10% w/v suspension. Tenfold serial dilutions of the lung suspensions were prepared, and 0.1 mL of each dilution was inoculated into the allantoic sac of five 10-day-old embryonated chicken eggs. Allantoic fluids from each egg were harvested 48 hr later and individually tested for hemagglutination activity. Human type O blood was obtained by venipuncture and placed into heparinized tubes. The red blood cells (RBC) were washed three times and resuspended to a final concentration of 0.75% in phosphate-buffered saline (PBS). The allantoic fluid (0.2 mL) was mixed thoroughly with 0.8 mL of PBS and 0.5 mL of the RBC suspension. The tubes were incubated at room temperature for 1 hr and then examined for hemagglutination. Those dilutions with positive hemagglutination were used in calculating the 50% egg infective dose (EID₅₀) by the method of Reed and Muensch (12).

Histopathology

On days 0, 3, 6, 9, and 12 following aerosol exposure, spleens were removed from four mice randomly selected from each of the six groups of mice (Flu, Flu/SAD, SAD, Flu/DMSO, DMSO, and untreated controls). Following inflation of the lungs with 10% formalin, the lungs and spleens were fixed in the formalin solution, dehydrated with ethanol, embedded in paraffin and sectioned into 6- μm sections. Sections were stained with hematoxylin and eosin and examined by light microscopy. Mice that were moribund or had just recently died were examined in terms of gross pathology and histopathology.

Hemagglutination Inhibition Assay

Blood was collected from mice in the Flu, Flu/SAD, SAD, and untreated control groups 21 days after aerosol exposure. Sera from five mice were pooled and heat inactivated at 56°C for 30 min. The pooled sera were diluted 1:5 in PBS. The diluted sera (0.5 mL) were mixed with 0.5 mL of 25% kaolin, an absorbant (Flow Laboratories, McLean, VA) and incubated at room temperature for 2 hr with occasional shaking. After centrifugation of the mixture at 1,000g for 10 min, the supernatant was removed and the serum incubated with 0.1 mL of packed human type O RBC for 1 hr at room temperature. The suspension was centrifuged and the supernatant removed. Twofold dilutions were made in V-bottom micro-

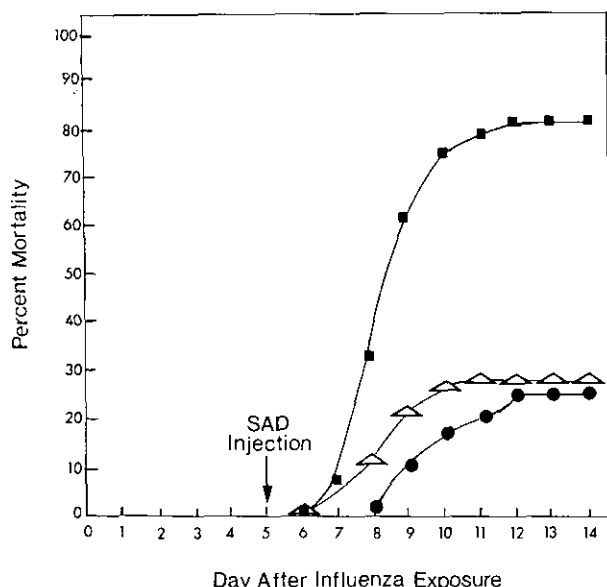


FIGURE 1. Cumulative mortality: (●)S group; (■)SF group; (△)F group.

titer plates such that each well contained 0.05 mL of diluted serum, 0.05 mL of diluted virus, and 0.05 mL of 1% human type O RBC. The plates were incubated at room temperature for 1 hr. Hemagglutination inhibition (HAI) titers were the reciprocal of the highest dilution of serum causing the inhibition of hemagglutination. The virus dilution used consisted of a 1:4 suspension of the hemagglutination titer.

Results

Mortality Studies

The mean time of death for the Flu and Flu/SAD groups of mice was 8.78 and 8.96 days, respectively, following virus aerosol exposure. The Kruskal-Wallis test indicated SAD administration to influenza infected mice did not significantly affect the mean time of death. Mice in the SAD only group had a mean death time of 5.52 days following SAD injection. This corresponded to day 10.52 after influenza virus exposure. The cumulative times of death are shown in Figure 1. Secalonic acid D administered alone resulted in 25% of the mice dying (15 of 60), whereas influenza alone produced a mortality rate of 28% (17 of 60). The two agents together (Flu/SAD) resulted in a mortality rate of 82% or 82 of 100 test mice. Using the chi-square test for two independent samples SAD significantly ($p < 0.001$) increased the mortality rate among influenza-infected mice.

Viral Pathogenesis

Influenza virus replication (increases in EID_{50} with time) was determined by titrating lung homogenates derived from each experimental group. The SAD, untreated control, and DMSO groups presented no evidence of influenza virus infection throughout the study period

Table 2. Virus titers (EID_{50}) in lung tissues.

Experimental group	Virus titer EID_{50} at various times after influenza virus exposure					
	0 days	3 days	5 days	6 days	9 days	12 days
Untreated control	0	0	a	0	0	0
DMSO			a	0	0	0
SAD only			a	0	0	0
Flu only	0	$10^{-6.9}$	a	$10^{-6.6}$	$10^{-2.5}$	0
Flu/DMSO			a	$10^{-5.5}$	$10^{-2.0}$	0
Flu/SAD			a	$10^{-6.5}$	$10^{-4.4}$	0

*On day 5 SAD or DMSO was administered.

(Table 2). This indicated that there was little or no cross contamination between cages. In mice exposed to virus aerosols, the virus titers peaked at 3 days after exposure. On day 5, DMSO was injected into infected mice (Flu/DMSO) and resulted in a slight decrease in virus titers on the following day. After day 6 similar declines in lung homogenate EID_{50} occurred in Flu and Flu/DMSO groups until day 12 when no virus was detected. Influenza-infected mice that were administered SAD (Flu/SAD) exhibited a slower recovery from virus infection as evidence by higher virus titers on days 6 and 9 than the Flu/DMSO group. Mice in this group (Flu/SAD) that survived to day 12 had no evidence of continuing viral replication in lung tissues.

Histopathology

Organ Weights. The weights of spleens and lungs as a percent of total body weights were determined for the mice in the different experimental groups (Table 3). No significant difference was evident in spleen weights among the various groups of mice at different times or with different treatment regimes. The weights of lungs on day 9 were significantly higher ($p > 0.025$) than those on days 6 and 12 for the virus-infected groups. There was also a significant increase ($p < 0.001$) in the weight of lungs between those groups exposed to influenza aerosols (Flu, Flu/SAD and Flu/DMSO) and those groups not exposed to influenza (untreated control, DMSO, and SAD) on days 6 and 9.

Lung Pathology. Mice from SAD, DMSO, and untreated control groups were generally free from lung lesions, although some background changes were noted. These background changes included a few small, focal paravascular or parabronchiolar lymphoid aggregates. Small areas of intraalveolar hemorrhage and atelectasis that were also present may have been artifacts produced at necropsy. No histopathologic differences were observed in lung tissues of Flu and untreated control group mice sacrificed on day 0. The Flu group by day 3 had extensive areas of necrotic bronchiolar epithelium and bronchiolar lumens were filled with necrotic cellular debris. Acute interstitial pneumonia was present in small areas. Alveolar spaces were partially collapsed and also contained necrotic cellular debris. Observations on day 6 after influenza exposure were similar to those seen 3 days after exposure, except that the epithelium remaining in the

Table 3. Weight of organs as percent of total body weight at various times after virus exposure.

Experimental group	Organ weight at various times after virus exposure ^a					
	Spleen			Lung		
	6 days	9 days	12 days	6 days	9 days ^b	12 days
Untreated control	0.42	0.35	0.37	0.82	0.91	0.78
DMSO	0.45	0.39	0.44	0.89	1.04	0.81
SAD	0.42	0.43	0.50	0.90	0.91	0.69
Flu	—	0.42	0.53	—	1.19 ^c	1.19 ^c
Flu/DMSO	0.54	0.55	0.47	1.33 ^c	1.78 ^c	1.29 ^c
Flu/SAD	0.51	0.37	0.47	1.09 ^c	1.64 ^c	1.28 ^c

^a No significant difference between treatment groups or times following virus exposure.

^b All mice exposed to influenza virus aerosols had significant increases in lung weights.

^c Day 9 mice exposed to influenza virus aerosols exhibited an increase in lung weights when compared to days 6 and 12.

bronchioles and alveolar lining cells were slightly hyperplastic. The interstitial pneumonia was more extensive and severe than was observed for day 3. On day 9, less necrosis of bronchiolar epithelium and bronchiolar inflammation were observed. The interstitial pneumonia was more chronic in appearance on day 9 than on day 6. Extensive alveolar lining cell and bronchial hyperplasia were dominant on day 12. The inflammation was resolving at this time, although there were areas of intraalveolar edema in Flu, Flu/DMSO, and Flu/SAD groups. There was no apparent histopathologic difference between mice in the Flu and Flu/SAD groups.

Spleens. Spleens from DMSO, SAD, and untreated control groups appear to be normal in all respects. Those spleens from mice in groups Flu, Flu/SAD, and Flu/DMSO showed increases in lymphoid hyperplasia and extramedullary hematopoiesis on days 9 and 12. However, there was no significant difference between the three groups in question.

Spontaneous Deaths. Histopathologic findings for the mice dying spontaneously or found to be moribund on days 6 and 7 after aerosol exposure (1 to 2 days after SAD administration) were similar to those mice sacrificed from the different groups in accordance with the study schedule described above. Pneumonia was severe and pulmonary edema was evident in those mice exposed to influenza aerosols. Mice administered SAD only exhibited minimal to moderate necrosis of the liver. Mice from the Flu/SAD group exhibited both pneumonia and liver necrosis (Fig. 2). Livers from mice in other groups were not examined. Three of the eleven mice receiving SAD possessed a dark reddish-brown fluid in their stomachs and small intestines.

Hemagglutination Inhibition Assay Results

The mean serum HAI titer for those mice exposed to virus only was 332, while the mean HAI titer for mice exposed to Flu and SAD (Flu/SAD) was 187. This Flu/SAD titer was significantly lower ($p < 0.05$) than that for mice of the Flu group. The HAI titer for mice from

the SAD group was 5.5. The control groups (DMSO and untreated controls) had no antibody response to the influenza virus whatsoever.

Discussion

There have been several reports which employ respiratory virus infections to study the role of environmental agents in altering the host's susceptibility to respiratory infections (13,14). Since SAD is a known contaminant of grain dusts (6), and has been reported to produce lung damage (5,7,9) experiments were designed to monitor effects SAD might have on the altered susceptibility of mice to influenza infections. This was important for the elucidation of the role of this grain dust contaminant in respiratory influenza infections.

Indeed, when SAD was administered to mice infected with influenza virus, an increase in mortality was observed. The resultant 82% mortality rate for the Flu/SAD group compared to the Flu rate of 28% and SAD rate of 25% indicated more than an additive effect between the two agents and suggested a possible synergistic effect. While the mortality rate increased with the combination of Flu and SAD the mean time of death for mice exposed to influenza virus aerosols was not altered. Animals in the Flu and Flu/SAD groups died approximately 9 days after aerosol exposure.

Assessment of virus replication in lung tissues revealed that the greatest virus replication occurred between days 3 and 6 after aerosol exposure and declined thereafter. Mice in the Flu/SAD group appeared to have higher virus titers than the Flu/DMSO group. But by day 12, all surviving animals showed no signs of residual virus in their lung tissues.

When the lungs from the different groups of mice were weighed it was found that the weights of the lungs obtained from mice exposed to influenza aerosols were much higher than the weight of the lungs from mice not exposed to influenza virus. The lung weights on day 9 after aerosol exposure were significantly higher ($p > 0.025$) than for days 6 and 12. The higher lung weights for the virus-infected mice could be attributed to edema. The pathological course of the influenza infection was characterized by maximal lung damage by day 9 followed by the initiation of the recovery process. No differences were observed between the extent or degree of infection in Flu, Flu/SAD, and Flu/DMSO groups of mice. Mice examined after spontaneous death 2 to 3 days post-SAD administration exhibited moderate liver necrosis in addition to the lung pathology, which was characteristic of influenza. Data from the present study suggest that the lung abnormalities previously reported for mice injected IP with SAD (5,7) may be only an artifact of necropsy.

There has been no evidence presented to date that related influenza infections to liver damage. Therefore, the liver pathology seen in the SAD treated mice and its similarity with the Flu/SAD groups were attributed to the effects of SAD alone and not due to its interaction with the influenza virus infection. It has been reported that the biotransformation of drugs by hepatic micro-

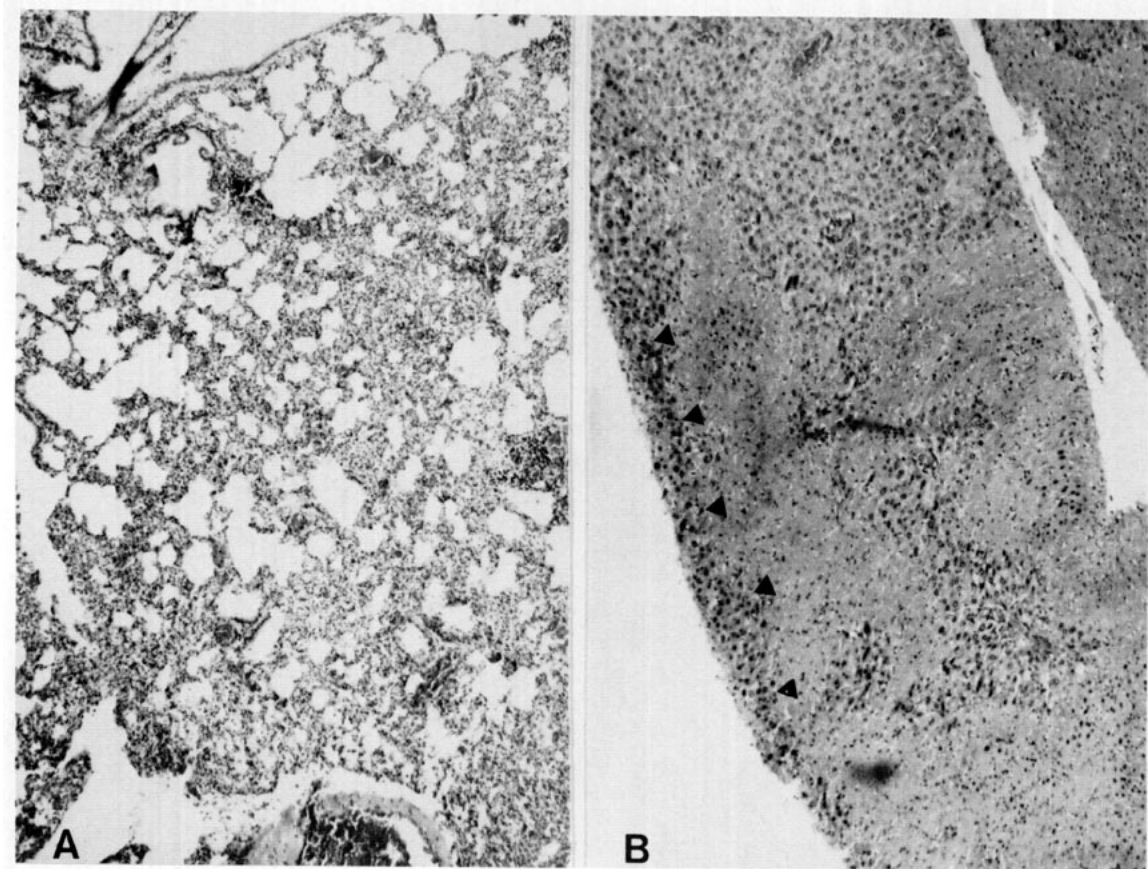


FIGURE 2. Histopathology observed in Flu/SAD mice dying 2 days after SAD administered: (a) lung tissue indicative of chronic pneumonia, magnified $\times 140$; (b) liver where arrows delineate normal from necrotic areas, magnified $\times 160$.

somes was decreased following stimulation of host defense mechanisms by a number of agents including interferon and viruses (15). Although the mechanism for SAD metabolism has not been elucidated it is thought that SAD is metabolized by the liver, since conjugates of SAD have been found in the bile. A decrease in SAD metabolism could possibly explain the elevated mortality rates for the Flu/SAD groups of mice.

Those mice dying from SAD (SAD and Flu/SAD groups) exhibited signs of mild tremors, lethargy, and withdrawal and cessation of eating and drinking. In these two groups, there were very few mice alive after days 9 and 12, which precluded the sampling and assaying of the prescribed number of mice as originally projected. However, it was observed that those mice surviving among the SAD or Flu/SAD groups did not exhibit overt signs of illness. The variability of responses to SAD observed in this study may indicate differences in the efficiency of individual mice to metabolize SAD. Reddy et al. (7) reported a wide range of LD_{50} values based on strain, sex, and age of the laboratory animal.

The HAI test results indicated that at 21 days after influenza virus aerosol exposure, there was a significant difference in the Flu and Flu/SAD groups' humoral immune responses. It is apparent that SAD significantly suppressed the antibody response to influenza virus in-

fection. Yamamoto (16) has previously reported that tumor cells exposed to SAD showed an inhibition of protein synthesis. A possible mechanism for the *in vivo* suppression of antibody formation observed in the Flu/SAD group may be explained by Yamamoto's *in vitro* findings.

Several studies have indicated that various mycotoxins may possess immunosuppressive properties (17-19). Any compromise in the immune system may result in an impairment of the host's ability to control microbial pathogens. This in turn could lead to infections and possible death of the host. Further studies to investigate the immunotoxic effects of SAD are currently underway.

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